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Production of a *Mycobacterium avium* ssp. *paratuberculosis* purified protein derivative (PPD) and evaluation of potency in guinea pigs

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Abstract. A *Mycobacterium avium* ssp. *paratuberculosis* purified protein derivative (PPD) was produced and the biologic activity evaluated in sensitized guinea pigs. The PPD when adjusted to a protein concentration of 1 mg/ml induced a delayed-type hypersensitivity response comparable to USDA Johnin OT 133-8707.

Key words: *Mycobacterium avium* ssp. *paratuberculosis*, PPD, delayed-type hypersensitivity.

Johne's disease is a contagious disease of cattle that causes major economic losses to the dairy and beef industry in the U.S. Although numerous *in vitro* and *in vivo* tests have been described for detection of animals infected with *Mycobacterium avium* ssp. *paratuberculosis* (Map), information on the standardization of antigen(s) used in the diagnostic tests is not available.[1] PPD and Johnin preparations currently available have been produced using a laboratory adapted strain of *M. avium*, referred to as Strain 18. Strain 18 is not representative of field strains of Map isolated from cattle.[2] No reference PPD antigen produced from a Map neotype strain is available in the U.S. In order to standardize diagnostic tests it is necessary to produce a PPD from a neotype strain of Map representative of field isolates.

The objectives of this study were to: (1) produce a reference PPD—using ATCC Map strain 19698, (2) determine the protein concentration of the PPD, (3) evaluate the biologic potency of the PPD in guinea pigs experimentally sensitized with Map.

Map (ATCC 19598) was subcultured on modified Reid's liquid medium for 60 days at 37° C to produce a seed culture. Five hundred millilitres of modified Reid's medium in 2 l Povitsky bottles was inoculated with the seed culture (3–5 loops of pellicle) and incubated for ten weeks at 37° C. Bottles were examined weekly for evidence of contamination.

Culture filtrate was prepared as previously reported.[3] Briefly, following incubation, bottles were autoclaved at 121° C for 30 min. After cooling overnight at 23° C, the cells were removed from the culture medium by straining through sterile 85 mesh nylon and gauze supported on stainless steel mesh to produce the culture filtrate (CF).

The proteins in the CF were precipitated by adding 40% trichloroacetic acid (TCA). The CF was mixed thoroughly while one part TCA was added to nine parts CF resulting in a solution with a final concentration of 4% TCA. This solution was allowed to stand overnight at room temperature without stirring. The next day, most of the supernatant was removed by aspiration and remaining supernatant was used to resuspend the precipitate and transfer to centrifuge bottles. The bottles were centrifuged at $2500 \times \boldsymbol{g}$ for 15 min to remove residual supernatant. The precipitate was washed twice with 1% TCA and once with 10% NaCl. The washed precipitate was then dissolved in solvent buffer (34 mM Na_2HPO_4, 86 mM NaCl) and the pH was adjusted to $7{\cdot}0 \pm 0{\cdot}1$ with 10 N NaOH. The solution was filter sterilized and kept in the dark at 4° C as a concentrate. The protein level of the PPD was determined by the micro-Kjeldahl method (protein = $6{\cdot}25 \times$ N).[3,4] The PPD was then adjusted to

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Table 1. Skin test responses of guinea pigs at 24 and 48 hours following injection of the current Johnin (133-8707) or the newly produced experimental purified protein derivative (PPD). Animals were sensitized with *M. avium* (Strain 18), the source of Johnin 133-8707 or with *M. avium* ssp. *paratuberculosis* (Strain 19698), the source of the experimental PPD and tested using three different dilutions of each antigen preparation

	Sensitinogen			
	M. avium (Strain 18)		*M. avium* ssp. *paratuberculosis* (Strain 19698)	
Antigen dose:	Johnin 133-8707*	PPD*	Johnin 133-8707*	PPD*
24 hour responses:				
0·02 mg	239·2 ± 15·6	238·4 ± 8·2	242·3 ± 9·6	244·6 ± 9·7
0·01 mg	167·1 ± 17·2	149·4 ± 14·1	171·2 ± 14·5	152·2 ± 17·5
0·005 mg	68·6 ± 6·0	61·4 ± 7·0	70·4 ± 4·0	61·1 ± 9·9
48 hour responses:				
0·02 mg	267·5 ± 18·7	259·6 ± 11·9	269·9 ± 12·7	260·5 ± 12·5
0·01 mg	187·5 ± 17·7	174·4 ± 15·5	191·3 ± 16·0	178·5 ± 13·2
0·005 mg	80·7 ± 6·1	76·8 ± 9·9	84·3 ± 5·2	75·9 ± 10·2

*Each cell is the mean response (mm^2 ± SEM) of ten guinea pigs.

1 mg/ml with sterile glucose phosphate dilution buffer (139 mM glucose, 28 mM Na_2HPO_4-$2H_2O$, and 19 mM KH_2PO_4). Phenol was added to the dilution buffer, as a preservative to a final concentration of 0·5%.

The PPD antigens were bioassayed in 10 guinea pigs sensitized with the Map type strain (ATCC 19698), and ten guinea pigs sensitized with *M. avium* strain 18. Two unsensitized guinea pigs served as controls. The guinea pigs were sensitized 35 days prior to the assay by injecting intramuscularly 0·5 ml of a sterile heat-killed suspension of the appropriate bacteria. PPD amounts of 0·02, 0·01, and 0·005 mg of protein were injected at separate sites. Each guinea pig received three dilutions of the experimental PPD and three dilutions of Johnin-133-8707. Reactions were examined at 24 and 48 h post challenge and the cross-sectional responses in mm were measured and recorded. Results (means) were analysed by ANOVA.

The culture filtrate yielded a solution that contained, by micro-Kjeldahl reaction, 18 mg protein/ml. Testing for purity and potency was done in accordance with USDA-APHIS guidelines in 9 CFR part 113.409.[5] There were no viable bacteria or fungi detected when the PPD was tested. Table 1 reports the results of skin tests in guinea pigs. Response means (erythema area in square millimetres) did not differ significantly due to the sensitizing strain or type of antigen tested (P<0·05). There were no statistical differences between observations at 24 or 48 h. The response difference due to antigen dose was significant; higher doses of antigen resulted in a larger response (P<0·05). No responses were observed to Johnin 133-8707 or the PPD antigen in guinea pigs not receiving sensitinogen. Dilutent alone did not result in a response in sensitized or unsensitized animals.

Johne's disease is characterized by a granulomatous inflammation of the posterior region of the small intestine, the ileocecal valve, the caecum and associated lymph nodes.[6] Clinical Johne's disease usually involves impaired intestinal function associated with chronic inflammatory responses; however these changes are not often observed in animals with subclinical disease.

Efforts to eliminate paratuberculosis in cattle has been limited by the lack of sensitive, reliable diagnostic tests for identifying all Map-infected animals. The use of an improved antigen (PPD) could increase the detection of cell-mediated responses in cattle infected with Map. The failure to detect animals in the early stages of disease is of particular importance to cattle producers interested in buying and selling breeding stock.

No reference PPD prepared from Map has been available for use in *in vitro* or *in vivo* diagnostic tests to detect cattle with clinical or subclinical Johne's disease. Since standardization of diagnostic tests is a high priority of the Johne's Disease

Committee of the United States Animal Health Association it is necessary to have a reference PPD antigen available to laboratories conducting tests. The new PPD, produced from a neotype strain, is of similar potency in guinea pigs as Johnin 133-8707. The PPD antigen(s) produced from a neotype strain of Map is currently under evaluation in cattle.

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